Defects in Rhizobial Cyclic Glucan and Lipopolysaccharide Synthesis Alter Legume Gene Expression During Nodule Development

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Submitted 15 June 2007. Accepted 4 October 2007.

cDNA array technology was used to compare transcriptome profiles of *Lotus japonicus* roots inoculated with a *Mesorhizobium loti* wild-type and two mutant strains affected in cyclic β (1-2) glucan synthesis (*cgs*) and in lipopolysaccharide synthesis (*lps* β 2). Expression of genes associated with the development of a fully functional nodule was significantly affected in plants inoculated with the *cgs* mutant. Array results also revealed that induction of marker genes for nodule development was delayed when plants were inoculated with the *lps* β 2 mutant. Quantitative real-time reverse-transcriptase polymerase chain reaction was used to quantify gene expression of a subset of genes involved in plant defense response, redox metabolism, or genes that encode for nodulins. The majority of the genes analyzed in this study were more highly expressed in roots inoculated with the wild type compared with those inoculated with the *cgs* mutant strain. Some of the genes exhibited a transient increase in transcript levels during intermediate steps of normal nodule development while others displayed induced expression during the final steps of nodule development. Ineffective nodules induced by the glucan mutant showed higher expression of phenylalanine ammonia lyase than wild-type nodules. Differences in expression pattern of genes involved in early recognition and signaling were observed in plants inoculated with the *M. loti* mutant strain affected in the synthesis of cyclic glucan.

Additional keywords: competitiveness, infection threads, *Lotus glaber*, Nod factor perception, phenolic compounds

Development of symbiotic associations between rhizobia and legumes involve a complex molecular communication, which leads to transcriptional reprogramming of both partners (Barnett et al. 2004; Colebatch et al. 2002a and b, 2004; El Yahyaoui et al. 2004; Kouchi et al. 2004). Plant-derived flavonoids play important roles during initial steps of this interaction. They are secreted by plant roots into the rhizosphere and induce production of bacterial lipochito-oligosaccharide compounds called Nod factors (Zuanazzi et al. 1998). Nod factor perception by the plant triggers physiological and morphological changes, including root hair deformation and initiation of cortical cell divisions leading to nodule primordium formation (Gage 2004). Simultaneously, bacteria are entrapped in an infection pocket at the inner site of the curled root hair and enter the plant cell via infection threads (ITs) that arise as a tubular in-growth bounded by plant cell wall (Brewin 2004). Bacteria grow and divide inside ITs while these progress toward the root cortex. Finally, bacteria are released from ITs into plant cells in developing nodules by an endocytotic process where they differentiate into nitrogen-fixing bacteroids (Gage 2004; Udvardi and Day 1997).

Many plant genes involved in root nodule formation and nitrogen fixation were discovered during the past years. Plant-symbiotic mutants allowed the identification of several loci involved in different steps of the nodulation process (Caetano-Anolles and Gresshoff 1991; Endre et al. 2002; Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003; Schauser et al. 1999; Stougaard 2000; Stracke et al. 2002; Szczygłowski et al. 1998). Among them were two LysM receptor-like kinase genes that were proposed to encode the putative Nod factor receptors in *Lotus japonicus* (*NFR1* and *NFR5*) (Madsen et al. 2003; Radutoiu et al. 2003) and in *Medicago truncatula* (*LYK3* and *LYK4*) (Limpens et al. 2003). Another receptor-like kinase gene mediating both mycorrhizal and rhizobial signals further downstream of the signaling cascade was described in *L. japonicus* (*SYMRK*) (Stracke et al. 2002) and in *M. truncatula* (*DMI2*) (Endre et al. 2002). Schauser and associates (1999) demonstrated that the *NIN* gene, encoding a putative transcription factor, is indispensable for IT formation and initiation of nodule primordia. Calcium spiking, a physiological phenomenon observed upon Nod factor perception, currently is believed to be mediated by the putative Ca-channel regulators *CASTOR/POLLUX* in *L. japonicus* and by *DMI1* in *M. truncatula* (Imaizumi-Anraku et al. 2005; Peiter et al. 2007).

Different approaches, including differential or subtractive hybridization techniques, led to the identification of several other nodule-specific or nodule-enhanced genes, named nodu-

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*The e-Xtra logo stands for "electronic extra" and indicates that three additional tables are published online.

lins (Cook et al. 1995; Dickstein et al. 1988; Gamas et al. 1996; Godiard et al. 2007; Jimenez-Zurdo et al. 2000; Szczygłowski et al. 1997).

More recently, large-scale expressed sequence tag (EST) and genome sequencing projects opened new perspectives for transcriptome research. Genes that are upregulated during symbiosis were predicted by *in silico* approaches (Fedorova et al. 2002; Journet et al. 2002; Lamblin et al. 2003; Tesfaye et al. 2006) or identified by using cDNA or oligonucleotide array technology (Barnett et al. 2004; Colebatch et al. 2002a and b, 2004; El Yahyaoui et al. 2004; Kouchi et al. 2004; Lohar et al. 2006; Mitra and Long 2004; Starker et al. 2006).

A series of bacterial mutants affected in polysaccharide synthesis were shown to be unable to infect plants successfully or to form functional bacteroids (Frayse et al. 2003; Lepek and D'Antuono 2005; Spaink 2000). Recently, we described the isolation and characterization of *M. loti* cyclic $\beta(1-2)$ glucan synthesis (*cgs*) and lipopolysaccharide synthesis (*lps* β 2) mutant strains (D'Antuono et al. 2005). The *cgs* mutant is unable to induce the formation of ITs. Although this strain was not affected in its capacity to trigger root hair curling and nodule formation, nodules that developed upon infection of *L. glaber* with this mutant were empty of bacteria (D'Antuono et al. 2005). The *lps* β 2 mutant synthesizes reduced amounts of O-antigen-containing lipopolysaccharide (LPS) molecules compared with the wild-type strain. It induced formation of nodules that were indistinguishable from those formed by the wild-type strain; however, co-inoculation experiments revealed that it was out-competed by the wild-type strain. The fact that the *M. loti lps* β 2 mutant induces a higher number of ITs than the wild type led to the hypothesis that it might have a competitive disadvantage either in being released from the ITs to the nodule cells or in its multiplication inside the nodule cells (D'Antuono et al., 2005).

Extensive cDNA array analysis of *L. japonicus* roots inoculated with both mutant and wild-type strains was used to study how the nodulation process is regulated at the transcriptional level. The aim of this study was to correlate some morphological and physiological changes that occurred during the nodulation process with changes in the plant transcriptome. A focus was put on the analysis of plant defense genes because a question still remains regarding the extent to which such systems are differentially regulated during infection, nodule organogenesis, and symbiotic nitrogen fixation.

RESULTS

Analysis of IT structures and bacterial release.

The nodulation phenotype of *cgs* and *lps* β 2 mutants on *L. glaber* was characterized recently (D'Antuono et al. 2005). Because the cDNA arrays used in this study were based on ESTs derived from *L. japonicus*, the nodulation phenotype on this plant genotype was studied before carrying out the cDNA array experiments.

L. japonicus roots inoculated with the *cgs* mutant developed small white nodules, as was observed on *L. glaber* (D'Antuono et al. 2005). No ITs could be found when *L. japonicus* was inoculated with a *cgs* mutant strain that express constitutively the green fluorescence protein (GFP) (data not shown).

In agreement with our previous results with *L. glaber*, the *lps* β 2 mutant strain induced the formation of wild-type fixing nodules on *L. japonicus*. Moreover, microscopic analyses of roots inoculated with a 1:1 mixture of *dsRED*-labeled *M. loti* wild-type and *GFP*-labeled *M. loti lps* β 2 strains revealed that both strains induced similar numbers of ITs (data not shown). After 20 days, plants developed normal and large fixing nodules; however, the recovery of the *lps* β 2 mutant strain from the

nodules was strongly reduced compared with the wild-type strain, with a competitiveness index of 10^{-4} (discussed below). These results support the previously formulated hypothesis that the affected step in *lps* β 2 mutant is the release or replication of the mutant inside nodule cells, and not IT formation (D'Antuono et al. 2005).

Identification of differentially expressed genes using cDNA arrays.

With the aim to understand how the nodulation process is regulated at a transcriptional level, transcriptome comparison between nodules and uninoculated *Lotus* roots was carried out previously (Colebatch et al. 2002a, 2004). This analysis also was made comparing inoculated roots at different postinoculation times with the uninoculated ones (Kouchi et al. 2004). Here, in order to correlate some morphological and physiological root changes with gene expression, we analyzed the transcriptome using bacterial mutants affected in some step of the nodulation for inoculation, and we related the results with gene expression differences observed between inoculated and uninoculated roots in a normal process.

To evaluate changes in gene expression during infection, RNA samples were obtained from roots inoculated with the wild-type or one of the two mutant strains at 7 and 28 days postinoculation (dpi) and from uninoculated and inoculated roots at 21 dpi. An enlarged version of the partially redundant cDNA array previously described (Colebatch et al. 2002a, 2004) was used. This array contained 9,600 EST clones derived from a mature and developing nodule EST library that were polymerase chain reaction (PCR) amplified and spotted onto nylon membranes (Ott et al. 2005). A significant differential expression value of twofold was used as a cut-off for further analysis. A complete list of genes can be found in Supplementary Table A. Examples of genes belonging to the different functional groups that were significantly induced in roots upon inoculation with the wild-type strain compared with the *cgs* mutant are shown in Table 1. Table 1 also shows the expression status in the comparison inoculated versus uninoculated roots at 21 dpi. The corresponding data were included in Supplementary Table B.

Most of the genes more highly expressed in plants inoculated with the wild type compared with the *cgs* mutant strain at 28 dpi are genes induced in nodulated roots compared with noninoculated roots at 21 dpi (Table 1). Furthermore, expression of these genes in roots inoculated with the wild-type strain is significantly higher at 28 than at 7 dpi (Table 1). Among these genes are several encoding transporters, transcription factors, and genes involved in nitrogen metabolism, signaling, and hormone metabolism. A majority of these genes was described earlier to be more highly expressed in nodules compared with uninoculated roots (Colebatch et al. 2004). In addition, several early and late nodulins were found to be more highly expressed in roots inoculated with the wild-type strain compared with those with the *cgs* mutant strain.

Genes involved in redox metabolism also were found to be overexpressed 28 dpi in roots inoculated with the wild-type compared with the *cgs* mutant. These data are in agreement with previous results describing the fact that functional nodules contain high antioxidative capacities (Matamoros et al. 2003) due to the requirement to detoxify reactive oxygen species that mainly derive from leghemoglobin auto-oxidation (Günther et al. in press).

A group of genes is overexpressed at early stages of nodulation (7 dpi) in plants inoculated with the wild-type strain compared, with the *cgs* mutant, but not at 28 dpi. Some of these genes such as those encoding a calcium channel α -1-I subunit, an aquaporin protein PIP1-1, a pyruvate decarboxylase, an

Table 1. Examples of differentially expressed *Lotus japonicus* genes, as determined by macroarray analysis, from a comparison between *Mesorhizobium loti* wild-type (wt) and cyclic β (1-2) glucan synthesis (*cgs*) mutant inoculations^a

Clone ID	TC no. ^b	Best database match ^c	Comparison wt and <i>cgs</i>				Roots ^d	Comparison wt at			
			7 dpi		28 dpi			28 and 7 dpi		7 and 28 dpi	
			Ratio	<i>P</i> value	Ratio	<i>P</i> value		Ratio	<i>P</i> value	Ratio	<i>P</i> value
29h12	14055	Nodulins									
90g2	14782	Leghemoglobin (BI419226)	5.546	0.0012	12.278	0.012	Induced	12.556	0.013	...	...
74f12	14488	Late nodulin Nlj16 [<i>Lotus japonicus</i>] (CB828802)	3.197	0.0025	15.749	0.041	Induced	12.366	0.043	...	...
100b2	8055	EARLY NODULIN 36A (CB827419)	2.318	0.0006	7.531	0.012	Induced	6.698	0.012	...	...
69f8	8309	Early nodulin ENOD18 [<i>Vicia faba</i>] (CB829623)	3.325	0.008	13.642	0.005	Induced	11.761	0.005	...	...
54a7	8588	Nlj21 [<i>Lotus japonicus</i>] (CB827047)	–	–	4.060	0.011	Induced	4.709	0.004	...	...
27b7	7989	Redox metabolism									
2g6	16336	Superoxide dismutase [<i>Cicer arietinum</i>] (BI420186)	9.022	0.002	–	–	WC	–	...	–	...
82b1	7860	Probable peroxidase (EC 1.11.1.7) (clone PC42) (BI418380)	4.019	0.006	–	–	Induced	–	...	–	...
70d7	8045	Oxidoreductase family [<i>Arabidopsis thaliana</i>] (AW719328)	–	–	20.870	0.006	Induced	36.925	0.005	...	...
88a3	Singleton	Proline oxidase precursor [<i>Arabidopsis thaliana</i>] (CB828043)	–	–	17.379	1.90 10 ^{–6}	Induced	18.828	1.954E-06	...	...
23h12	7806	Transport									
56d8	15862	Sulfate transporter-related [<i>Arabidopsis thaliana</i>] (CB827109)	–	–	34.995	0.008	Induced	28.701	0.008	...	...
96d10	16504	Calcium channel α-1-I subunit [<i>Rattus norvegicus</i>] (CB828566)	8.635	0.002	–	–	WC	...	...	4.417	0.033
92f5	Singleton	Aquaporin protein PIP1;1 [<i>Medicago truncatula</i>] (BI418198)	5.220	1.841	–	–	Repressed	...	...	3.472	1.016E-05
19f11	16363	Aquaporin-like transmembrane channel protein (BI420390)	3.760	0.0006	–	–	Repressed	...	...	3.833	0.006
83c5	Singleton	Sorbitol-like transporter [<i>Glycine max</i>] (CB829289)	3.202	0.011	11.745	0.009	Induced	9.094	0.009	...	...
37g5	7822	Potassium transporter, putative (KUP3/KT4/POT4) [<i>Arabidopsis thaliana</i>] (CB828967.1)	3.032	0.009	–	–	Repressed	...	...	...	...
37f8	9381	Sucrose transporter 4 protein (AW720468)	4.200	0.011	–	–	Repressed	...	...	2.298	0.027
67h4	13869	Mannitol transporter, putative [<i>Arabidopsis thaliana</i>] (CB828147)	–	–	4.369	0.012	Induced	4.642	0.010	...	...
41h1	13869	CF0 ATP synthase:SUBUNIT=9 (BI417853)	5.795	0.009	–	–	WC	...	...	3.084	0.035
69e5	12768	Response to pathogens and other external factors									
50f4	14348	PAL (BI417846)	2.013	0.008	–	–	WC	–	...	–	...
84f5	Singleton	PAL (CB826882)	–	–	13.165	0.019	Induced	10.620	0.018	...	...
37d2	10233	Avr9/Cf-9 rapidly elicited protein 31 [<i>Nicotiana tabacum</i>] (BI419590)	3.656	0.021	–	–	WC	–	...	–	...
31a6	8688	Avr9/Cf-9 rapidly elicited protein 264 [<i>Nicotiana tabacum</i>] (CB827033)	–	–	6.908	0.023	Induced	7.333	0.023	...	...
32c9	15071	Snakin-1 [<i>Solanum tuberosum</i>] (BI418686)	3.080	0.038	–	–	WC	–	...	–	...
85g11	14744	NLS-TIR-NBS disease resistance Protein [<i>Populus tremula</i>] (CB828263)	2.415	0.020	–	–	WC	–	...	–	...
		NtPRp27-like protein [<i>Solanum tuberosum</i>] (BI417829)	5.081	0.003	–	–	Repressed	–	...	–	...
		Endo-1,4-β-D-glucanase [<i>Pyrus communis</i>] (BI417870)	2.341	0.011	–	–	Induced	–	...	–	...
		Disease resistance response protein-related/dirigent protein-related (DRRP) (BI417979)	2.563	0.019	–	–	Repressed	–	...	–	...
		Cytochrome P450 71D11 (CB828362)	–	–	2.907	0.016	Induced	–	...	–	...

(continued on following page)

^a Expression ratios are given for comparisons of different strain inoculations and different timepoints. A selection of ratios higher than twofold with *P* value, obtained from Student *t* test, ≤ 0.05 is shown; dpi = days postinoculation.

^b TC = tentative consensus sequence.

^c GenBank accession numbers are given in parenthesis. Phenylalanine ammonia-lyase = PAL.

^d Comparison of inoculated and uninoculated roots at 21 dpi. WC = without change.

Table 1. (continued from preceding page)

Clone ID	TC no. ^b	Best database match ^c	Comparison wt and cgs				Roots ^d	Comparison wt at			
			7 dpi		28 dpi			28 and 7 dpi		7 and 28 dpi	
			Ratio	P value	Ratio	P value		Ratio	P value	Ratio	P value
88g6	19604	Wound-induced protein–tomato (fragment) (CB828625)	4.000	0.006	–	–	WC	–	...	–	...
67c2	14525	Isoliquiritigenin 2'-O-methyltransferase–alfalfa >gi 13399462 (CB826820.1)	2.894	0.049	6.216	0.001	Induced	–	...	–	...
77b3	14197	Hormone related Auxin-repressed protein [<i>Robinia pseudoacacia</i>] (CB827639)	–	–	7.301	0.002	Induced	8.852	0.002	...	...
92b9	14714	INDOLE-3-ACETIC ACID INDUCED PROTEIN ARG2 (CB828926)	–	–	6.135	0.001	Induced	3.719	0.001	...	...
92d4	14222	1-Aminocyclopropane-1-carboxylate oxidase (ACC oxidase) (ethylene-forming enzyme) (CB828944)	–	–	15.410	0.015	Induced	11.707	0.015	...	...
74e11	8520	Transcription Tuber-specific and sucrose-responsive element binding factor (CB827409)	5.462	0.013	7.219	0.012	Induced	8.752	0.011	...	...
97g10	14538	CCCH-type zinc finger protein-related [<i>Arabidopsis thaliana</i>] (CB829404)	3.867	0.012	14.153	0.032	Induced	–	...	–	...
76g8	Singleton	DHHC-type zinc finger domain-containing protein [<i>Arabidopsis thaliana</i>] (CB827611)	2.132	0.0004	5.902	0.008	Induced	–	...	–	...
40f11	18247	KH domain protein [<i>Arabidopsis thaliana</i>] (BI419487)	3.895	0.001	–	–	WC	–	...	–	...
85d2	14104	Other metabolisms Asparagine synthetase [glutaminehydrolyzing] 2 (CB828330)	–	–	12.492	0.004	Induced	10.013	0.004	...	...
90c4	8402	Glyceraldehyde 3-phosphate dehydrogenase, cytosolic (CB828758)	5.844	0.010	10.806	0.003	Induced	–	...	–	...
93g5	14491	Aspartate aminotransferase [<i>Lotus corniculatus</i> var. <i>japonicus</i>] (CB829072)	–	–	9.575	0.002	Induced	–	...	–	...
12e4	7962	Alcohol dehydrogenase 1; ADH1 [<i>Lotus corniculatus</i>] (AW719956)	8.017	0.002	–	–	Repressed	–	...	–	...
76f3	14068	Thiamin biosynthetic enzyme [<i>Glycine max</i>] (CB827595)	7.611	0.005	–	–	WC	–	...	–	...
64b1	7922	Pyruvate decarboxylase 1 [<i>Lotus corniculatus</i>] (BI421001)	3.991	0.036	–	–	Repressed	...	...	3.293	0.047
41b12	9707	Sterol delta-7 reductase (BI4°9531.1)	4.063	0.009	–	–	Repressed	...	...	...	...
88g9	14425	Putative chorismate mutase precursor [<i>Oryza sativa</i> (japonica cultivar-group)] (BI419531)	–	–	13.072	0.007	Induced	12.195	0.007	...	...
15h7	8009	Cytosolic phosphoglycerate kinase [<i>Pisum sativum</i>] (AW720663)	3.424	0.016	8.956	0.009	Induced	–	...	–	...
18b1	10832	Arginine/serine-rich protein, putative (AW720583)	3.799	0.0004	0.674	0.043	Repressed	...	...	3.621	0.0004
60e1	14075	Cell growth and wall synthesis Hydroxyproline-rich protein–soybean (BI420695)	3.506	0.015	–	–	Repressed	–	...	–	...
32e11	8787	Putative extensin [<i>Oryza sativa</i>] (BI417994)	6.245	0.006	–	–	WC	–	...	–	...
22g8	14476	TUBULIN β-1 CHAIN (AW720428)	3.049	0.001	–	–	Repressed	...	...	2.059	0.004
23h2	15125	Signaling EST AU070346(S12172) corresponds to a region of the predicted gene. Approximately similar to AMP-binding protein (BI418199)	5.722	0.016	–	–	Repressed	...	...	4.902	0.018
87d3	16248	Receptor protein kinase-like protein [imported]–[<i>Arabidopsis thaliana</i>] (CB828513)	3.199	0.007	8.815	0.002	Induced	–	...	–	...
12h3	8589	RAB5B [<i>Lotus corniculatus</i> var. <i>japonicus</i>] (AW719977)	6.539	0.009	–	–	WC	–	...	–	...

arginine/serine-rich protein, a sterol δ -7 reductase, a superoxide dismutase, a probable peroxidase, a tubulin β -1 chain, and a RAB5B, are repressed or not significantly altered when comparing wild-type inoculated with noninoculated control plants at 21 dpi (Table 1). Expression of some of these genes also was detected as being significantly higher at 7 than at 28 dpi in the wild-type inoculation. These results suggest that those genes display a transient induction during nodulation, a process not occurring in plants inoculated with the *cgs* mutant.

Global comparison of root gene expression profiles 7 dpi with both mutants revealed similar significant underexpression of genes involved in sugar and protein metabolism, transcription, redox metabolism, response to pathogens, hormonal response, and transport, as well as some encoding for nodulins compared with roots inoculated with the wild-type strain. At 28 dpi, the differences were much less pronounced in *lps* β 2 mutant- than in *cgs* mutant-inoculated plants.

Kinetic analysis of nodulin, defense-, and redox-related genes during nodulation.

Array analysis at 7 and 28 dpi revealed that expression of a number of genes depends on the bacterial genotype and on the time after inoculation. Among them were genes putatively involved in redox metabolism and plant defense response. It remains to be elucidated to what extent plant defense responses are triggered during nodulation; therefore, several genes were studied in more detail using quantitative reverse-transcriptase (qRT)-PCR. Genes involved in these processes were chosen based on their differential expression observed in transcriptome analysis (Table 2). To get a more complete view, other genes described in independent studies to be involved in defense responses were included in this analysis. Among them were genes encoding a resistant-specific protein-3 (*RES3*) (TC 8082), a pathogenesis-related protein PR10-1 (*PR10-1*) (TC 15006), a putative flavanone 3-hydroxylase (*F3H*) (TC 7931), a peroxidase (*PX*) (TC 14066), a gibberellin-2 oxidase (*GA-2 OXIDASE*) (TC 8129), and a putative WRKY transcription

factor NtSub-D48 (*NTSUB-D48*) (TC 9957) (Table 2). Expression levels were studied at 7, 14, and 21 dpi in roots of plants inoculated with the wild-type or the mutant strains.

NLJ21 was significantly induced in roots of plants inoculated with the wild-type or the *lps* β 2 strain, with the highest expression detected 21 dpi. Such induction was not found in roots inoculated with the *cgs* mutant (Table 2). Additionally, significantly increased transcript levels of gene encoding superoxide dismutase (*SOD*) were observed in plants inoculated with the wild-type strain during intermediate time of nodulation, whereas no induction was detected in plants inoculated with the *cgs* mutant (Table 2). On the other hand, expression levels of the genes encoding phenylalanine ammonia lyase (*PAL*) and NtSub-D48 were more highly expressed in roots inoculated with the *cgs* mutant than with the wild-type or *lps* β 2 mutant. These results reveal some of the molecular differences comparing nodule development between the wild-type and the *cgs* mutant.

Based on the results presented in Table 2, genes were divided into two major groups according to their temporal expression pattern throughout the symbiotic process. The first group comprised genes that are induced in mature nodules (21 dpi) hosting wild-type bacteria. This pattern was found for genes encoding NlJ21, ENOD40, GA-2 oxidase, snakin-1, PAL, and F3H. These genes, except PAL, were not expressed in nodule-like structures induced by the *cgs* mutant. A second group comprised genes that displayed maximum expression in immature, developing nodules. These included those encoding SOD, RES 3, endo-1,4- β -D-glucanase, peroxidase, NLS Toll-interleukin receptor nucleotide binding site (TIR-NBS) disease resistance protein, NtPRp27-like protein, and PR10-1. None of these genes was found to be induced in plants inoculated with the *cgs* mutant. Expression patterns of *SOD*, *NLS-TIR-NBS*, and a gene encoding endo-1,4- β -D-glucanase were similar in plants inoculated with the *lps* β 2 mutant compared with the wild-type strain, although at lower levels. On the other hand, expression levels of *PX*,

Table 2. Expression levels were determined by quantitative reverse-transcriptase polymerase chain reaction and calculated as ratios compared with uninoculated control roots

Gene	Relative transcript level								
	7 dpi			14 dpi			21 dpi		
	<i>cgs</i>	<i>lps</i>	wt	<i>cgs</i>	<i>lps</i>	wt	<i>cgs</i>	<i>lps</i>	wt
<i>NLJ21</i> (CB827047)	1.16 $\pm$ 0.11	0.34 $\pm$ 0.07	0.52 $\pm$ 1E ⁰⁴	1.15 $\pm$ 0.14	0.88 $\pm$ 0.40	2.00 $\pm$ 0.98	1.41 $\pm$ 0.46	2.94 $\pm$ 0.29	4.09 $\pm$ 0.75
<i>ENOD40</i> (CB827419)	0.83	1.04 $\pm$ 0.03	0.91 $\pm$ 0.14	0.66 $\pm$ 0.06	1.50 $\pm$ 0.37	1.33 $\pm$ 0.31	1.09 $\pm$ 0.30	2.85 $\pm$ 0.51	4.29 $\pm$ 0.30
Endoglucanase-encoding gene (BI417870)	0.61 $\pm$ 0.02	0.45 $\pm$ 0.02	0.61 $\pm$ 0.02	1.25 $\pm$ 0.44	2.20 $\pm$ 0.21	3.52 $\pm$ 0.54	1.73 $\pm$ 0.07	1.65 $\pm$ 0.07	1.11 $\pm$ 0.38
<i>RES3</i> (CB827541)	0.41 $\pm$ 0.01	0.49 $\pm$ 0.01	0.64 $\pm$ 0.01	0.85 $\pm$ 0.05	0.98 $\pm$ 0.07	2.31 $\pm$ 0.34	1.14 $\pm$ 0.01	1.36 $\pm$ 0.01	1.13 $\pm$ 0.22
<i>NLS</i> (CB828263)	0.57 $\pm$ 0.02	1.11 $\pm$ 0.14	0.92 $\pm$ 0.06	1.32 $\pm$ 0.20	2.11 $\pm$ 0.11	2.45 $\pm$ 0.57	1.49 $\pm$ 0.26	1.52 $\pm$ 0.03	1.21 $\pm$ 0.28
<i>DRRP</i> (BI417979)	1.44 $\pm$ 0.03	1.72 $\pm$ 0.01	1.62 $\pm$ 0.03	0.71 $\pm$ 0.12	0.87 $\pm$ 0.12	0.73 $\pm$ 0.11	0.72 $\pm$ 0.01	0.78 $\pm$ 0.05	0.48 $\pm$ 0.05
<i>SNKIN-1</i> (BI418686)	0.50 $\pm$ 0.05	1.03 $\pm$ 0.02	0.98 $\pm$ 0.02	0.57 $\pm$ 0.07	1.19 $\pm$ 0.06	1.50 $\pm$ 0.42	1.02 $\pm$ 0.07	1.91 $\pm$ 0.07	2.11 $\pm$ 0.98
<i>PR10</i> -(BI417503.1)	0.36 $\pm$ 0.01	0.71 $\pm$ 0.03	0.82 $\pm$ 0.04	0.94 $\pm$ 0.32	1.18 $\pm$ 0.15	2.14 $\pm$ 0.50	1.63 $\pm$ 0.03	1.59 $\pm$ 0.04	0.97 $\pm$ 0.06
<i>NTPRP27</i> (BI417829)	0.06	0.83 $\pm$ 0.05	1.26 $\pm$ 0.08	1.40 $\pm$ 0.56	1.41 $\pm$ 0.61	5.84 $\pm$ 2.02	1.88 $\pm$ 0.46	2.28 $\pm$ 0.14	1.15 $\pm$ 0.36
<i>F3H</i> (BI419517)	0.58 $\pm$ 0.04	2.20 $\pm$ 0.10	2.21 $\pm$ 0.51	2.00 $\pm$ 0.56	2.62 $\pm$ 0.62	4.34 $\pm$ 1.44	3.76 $\pm$ 0.71	13.72 $\pm$ 4.06	12.03 $\pm$ 4.96
<i>SOD</i> (BI420186)	0.34 $\pm$ 0.00	0.63 $\pm$ 0.01	0.79 $\pm$ 0.03	1.23 $\pm$ 0.39	1.92 $\pm$ 0.37	3.80 $\pm$ 0.29	0.90 $\pm$ 0.03	1.12 $\pm$ 0.04	0.65 $\pm$ 0.07
<i>PX</i> (BI418564.1)	0.39 $\pm$ 0.02	0.56 $\pm$ 0.00	0.77 $\pm$ 0.08	1.25 $\pm$ 0.40	1.09 $\pm$ 0.16	2.19 $\pm$ 0.22	1.18 $\pm$ 0.01	1.61 $\pm$ 0.02	1.15 $\pm$ 0.09
<i>GA 2-OXIDASE</i> (BI420933.1)	0.36 $\pm$ 0.00	2.25 $\pm$ 0.53	1.54 $\pm$ 0.09	3.61 $\pm$ 0.72	3.62 $\pm$ 0.27	3.32 $\pm$ 1.32	2.75 $\pm$ 0.88	14.31 $\pm$ 1.65	20.97 $\pm$ 1.43
<i>PAL</i> (BI417846)	0.88 $\pm$ 0.04	0.66 $\pm$ 0.09	0.39 $\pm$ 0.25	1.40 $\pm$ 0.54	1.60 $\pm$ 0.68	2.87 $\pm$ 0.56	11.31 $\pm$ 3.31	1.85 $\pm$ 0.51	3.01 $\pm$ 1.17
<i>NTSUB-D48</i> (BI417354.1)	0.35 $\pm$ 0.05	0.63 $\pm$ 0.10	0.81 $\pm$ 0.15	1.39 $\pm$ 0.21	1.88 $\pm$ 0.16	1.47 $\pm$ 0.07	3.24 $\pm$ 0.25	1.57 $\pm$ 0.09	1.11 $\pm$ 0.65

^a Data were normalized to ubiquitin. Abbreviations: dpi = days postinoculation, *cgs* = cyclic β (1-2) glucan synthesis mutant, *lps* = lipopolysaccharide synthesis mutant, and wt = wild-type strain. Maximal expression levels for each strain are in bold.

^b GenBank accession numbers are given in parentheses.

RES3, and the NtPRp27-encoding gene (*NTPRP27*) in plants inoculated with the *lpsβ2* mutant were similar to those observed in plants inoculated with the *cgs* mutant.

In general, results obtained with qRT-PCR confirmed those obtained with cDNA array analysis. Only a few divergences were found. For example, using cDNA arrays, *DRRP* was found to be twofold higher expressed in wild-type-inoculated roots than in roots infected with the *cgs* mutant (Table 1). However, no significant differences were found by qRT-PCR (Table 2). Some of the divergences might be explained by the sensitivity and the higher degree of specificity of qRT-PCR methodology. Thus, expression data for genes being members of large gene families with high degrees of sequence conservation have to be considered more cautiously. Therefore, discrimination between expression levels of highly similar genes might be difficult.

Accumulation of phenolic compounds in roots and nodules.

As reported above, a gene encoding a PAL was found to be strongly induced 21 dpi with the *cgs* mutant. PAL is a key enzyme involved in the production of phenolic compounds (Baron and Zambryski 1995). To test whether such compounds were accumulated in nodule-like structures induced by the *cgs* mutant, roots were treated with potassium permanganate and were examined using light microscopy at different postinoculation times (7, 15, and 28 days) (Fig. 1). At 7 and 15 dpi, roots inoculated with the *cgs* mutant showed strong stained regions, indicating the presence and accumulation of polyphenolic compounds (Fig. 1). In contrast, no phenolic compounds were observed in roots that were inoculated with the wild-type strain or in noninoculated control roots. Later during the course of nodulation (28 dpi), homogeneous brown staining was found in plants inoculated with the wild-type and *cgs* mutant; however, accumulation of phenolic compounds was significantly higher in roots inoculated with the *cgs* mutant strain than in roots inoculated with the wild-type strain (Fig. 1). Such enrichment also was observed in nodule-like structures induced by the *cgs* mutant (Fig. 1).

Early nodule gene expression.

As reported above, expression of several early nodulins was altered in roots inoculated with the *cgs* mutant strain. Nodule-like structures that developed upon inoculation of roots with the *cgs* mutant did not harbor any bacteria. Because the mutant produces Nod factors normally (data not shown), we examined whether expression patterns of genes involved in early signaling events (*NFR5*, *SYMRK*, *NIN*, *POLLUX*, *CASTOR*, and *ENOD2*) were altered. For these experiments, RNA was extracted from roots 12, 24, and 48 h postinoculation (hpi) and 4, 7, 14, and 21 dpi.

No significant difference between strains was observed for the expression of *NFR5*, *SYMRK*, and *CASTOR* during nodule induction (Fig. 2), whereas *POLLUX* showed induced expression in roots inoculated with the *cgs* mutant 12 hpi. Similar transcript levels using wild-type rhizobia were observed only at 14 dpi (Fig. 2).

The *NIN* gene encodes a putative transcription factor that is required for the formation of ITs and initiation of primordia (Schauser et al. 1999). According to previous reports (Radutoiu et al. 2003; Schauser et al. 1999), *NIN* shows an upregulation during the nodulation process induced by the wild-type strain. No transcriptional activation of *NIN* was observed in roots inoculated with the *cgs* mutant (Fig. 2).

ENOD2 expression was described to be increased upon initiation of nodule cell differentiation and to require a functional *NFR1* and *NFR5* (Radutoiu et al. 2003). Analysis of *ENOD2* gene expression revealed that the strong increment observed in roots inoculated with the wild-type strain between 7 and 21 dpi was not detected in roots inoculated with the *cgs* mutant strain (Fig. 2).

DISCUSSION

cDNA array analysis.

cDNA array analysis of plant genes was used to elucidate molecular differences between roots inoculated with wild-type and two rhizobial mutant strains (*cgs* and *lpsβ2*).

Fig. 1. Accumulation of phenolics compounds in roots and nodules of *Lotus japonicus*. Uninoculated and inoculated plants with the different strains of *Mesorhizobium loti* (wild-type [wt] or cyclic $\beta(1-2)$ glucan synthesis [*cgs*] mutant) were harvested at several timepoints postinoculation (days postinoculation [dpi]), were treated with potassium permanganate, and were examined using light microscopy. Scale bar indicates 250 μ m.

Although induction of defense- and stress-responsive pathways during symbiotic interactions has been reported previously (Colebatch et al. 2004; El Yahyaoui et al. 2004; Kouchi et al. 2004; Liu et al. 2003; Manthey et al. 2004; Niehaus et al. 1993; Ramu et al. 2002; Santos et al. 2001; Vasse et al. 1993), the use of bacterial mutants enabled us to study expression of these systems in more detail.

Transcript profiles of roots 7 and 28 dpi revealed several genes to display increased expression levels in roots inoculated with the wild-type strain compared with those inoculated with the *cgs* mutant strain. Considering the defective nodule phenotype induced by the *cgs* mutant, we speculate that genes that lack induction by the mutant may be genes involved in nodule maturation. Consistent with this idea, most of these genes were found to be induced in roots with mature nodules containing wild-type rhizobia compared with uninoculated roots. Array results obtained at different time point postinoculation also suggest the existence of transient expression of some of the identified genes.

Fewer and generally less pronounced changes were observed in roots of plants inoculated with the *lpsβ2* mutant 28 dpi compared with the wild-type. These results are in agreement with

the nodulation phenotype of this mutant that induces the formation of wild-type-like nodules. However, there is a delay of the expression of several genes at 7 dpi. Because *lpsβ2* displays no defect on the formation of ITs, it can be assumed that most of the genes differentially regulated might play roles in cell invasion or in the successive events of nodule development rather than in earlier steps of infection.

Gene expression during the nodulation process analyzed by real-time RT-PCR.

Nodulins, defense-, and redox-related genes. Genes with constantly increased expression during nodule development (*ENOD40*, *GA-2 OXIDASE*, *F3H*, *SNAKIN-1*, and *NLJ21*) were grouped. Expression of these genes seems to be related to the development of a fully active nodule because most of them were not expressed or displayed low expression in the inactive nodules formed by the *cgs* mutant.

GA-2 oxidase and F3H are involved in plant defense responses and were described previously as strongly induced in mature nodules (Kouchi et al. 2004). Nlj21, a glutamate-rich nodulin protein, also was associated with late nodule developmental events during nodule organogenesis in *L. japonicus*

Fig. 2. Real-time reverse-transcriptase polymerase chain reaction expression analysis of transcript levels of plant genes involved in Nod factor perception and early signaling. **A**, *NFR5*. **B**, *SYMRE*. **C**, *POLLUX*. **D**, *CASTOR*. **E**, *ENOD2*. and **F**, *NIN* expression was determined at different times postinoculation in roots of plants inoculated with *Mesorhizobium loti* wild-type (wt) strain (black bars) or with cyclic β (1-2) glucan synthesis (*cgs*) mutant strain (gray bars). Expression levels are shown relative to un-infected roots at the same time point. The error bars indicate standard deviation.

(Szczyglowski et al. 1998). Both *GA-2 OXIDASE* and *NLJ21* expression were described to be repressed in ineffective *sen1* nodules (Suganuma et al. 2003).

Our results support the hypothesis that these two proteins are strictly associated with an active nodule because their expression was not induced in nonfunctional nodules formed by the *cgs* mutant.

Snakins form a large family of antimicrobial peptides in plants (Broekaert et al. 1995; Garcia-Olmedo et al. 1998, 2001). Among them, snakin-1 and -2 exhibited strong antimicrobial activity in potato plants (Berrocal-Lobo et al. 2002; Segura et al. 1999). Here, we report for the first time that snakin-1 also is induced during a symbiotic interaction. This gene was not found to be induced in roots that were inoculated with the *cgs* mutant; therefore, it can be suggested that such peptides might be involved in control of infection once plant cell invasion occurs.

A second group of genes exhibited a transient induction during intermediate stages of nodule development and a subsequent decrease. This group included several other defense (*PR10-1*, *NLS-TIR-NBS*, *RES3*, endo- β -1,4-glucanase-encoding gene, and *NTPRP27*) and redox (*PX* and *SOD*)-related genes. Increased expression of *PR10-1* and *NTPRP27* was reported previously during plant-pathogen interactions (Hadwiger et al. 1992; Liu et al. 2003; Okushima et al. 2000), and expression of a gene encoding for a putative endo- β -1,4-glucanase from *M. truncatula* was found to be induced in arbuscular mycorrhizal symbiosis (Liu et al. 2003). Also, an EST similar to endo- β -1,4-glucanase was found to accumulate in roots of *M. truncatula* during root symbiosis (Manthey et al. 2004).

A number of defense- and stress-related genes previously were reported to display transient expression during the *L. japonicus*-*M. loti* interaction (Kouchi et al. 2004) and during arbuscular mycorrhizal symbiosis in *M. truncatula* (Liu et al. 2003). Here, we found that, during the development of empty nodules induced by the *cgs* mutant strain, transient induction of the majority of the defense-related or stress-related genes did not occur.

In the inoculation with the *lpsb2* mutant, expression of *RES3*, *NTPRP27*, and *PX* were reduced drastically when compared with wild-type inoculations. Although this suggests that regulation of these genes is linked to bacterial invasion, a direct role of LPS O-antigen on its induction cannot be ruled out.

Another enzyme frequently associated with plant host defense and plant stress is PAL (Pallas et al. 1996). PAL accumulation previously was correlated with the infection process in the *Sinorhizobium meliloti*-alfalfa interaction (Vasse et al. 1993). This enzyme is involved in the synthesis of phenolic compounds and an increased level of these molecules was observed in pseudonodules induced by *S. meliloti* EPSI⁻ mutants (Niehaus et al. 1993).

Some increment of *PAL* expression was observed in roots inoculated with the wild-type strain; however, its expression was strongly induced in roots of plants inoculated with the *cgs* mutant. Invading microorganisms have developed specialized mechanisms to modulate or avoid host defense responses (Chisholm et al. 2006). In rhizobia, bacterial polysaccharides, effectors proteins, and Nod factors were postulated to fulfill these functions (Bartsev et al. 2004; Bhagwat et al. 1999; Fraysee et al. 2003; Mitra and Long 2004; Perotto et al. 1994). Low levels of *PAL* expression in roots inoculated with the wild-type strain compared with the *cgs* mutant suggests that *PAL* expression is controlled during wild-type interactions, a mechanism that is absent in the *cgs* mutant interaction. It was described that LPS from *S. meliloti* was able to suppress expression of several *M. truncatula* genes related to the plant response, including *PAL* (Tellstrom et al. 2007). Our experi-

ments indicate that the presence of the bacterial components inside the ITs might be required for suppression of *PAL* expression.

Genes involved in Nod factor perception and signal transduction. Several genes involved in Nod factor perception and early signal transduction that are essential for nodule organogenesis in *L. japonicus* have been cloned. Among them were the putative Nod factor receptor genes *NFR1* and *NFR5* (Madsen et al. 2003; Radutoiu et al. 2003); the symbiotic receptor-like kinase gene *SYMRK* (Stracke et al. 2002); *NIN*, a gene encoding a putative transcription factor required for IT formation and inception of the nodule primordia (Schauser et al. 1999); and genes such as *POLLUX* and *CASTOR* that were predicted to encode ion channels (Imaizumi-Anraku et al. 2005).

Although expression of *NFR5*, *SYMRK*, and *CASTOR* appeared not to be altered in roots inoculated with the *cgs* mutant, expression of *POLLUX* was significantly higher in roots inoculated with the *cgs* mutant than in roots inoculated with the wild-type strain. The results support earlier findings that expression of *POLLUX* is controlled by the bacteria (Imaizumi-Anraku et al. 2005).

Another significant difference was found for the putative transcription factor *NIN*. In agreement with earlier results (Radutoiu et al. 2003), we observed *NIN* expression to be continuously increased during nodulation process. Such behavior was not observed in roots inoculated with the *cgs* mutant. Previous publications indicated that application of purified Nod factors on seedlings led to induction of *NIN* expression and negative regulation of *POLLUX* transcription (Imaizumi-Anraku et al. 2005; Radutoiu et al. 2003). The *cgs* mutant normally secretes Nod factors and is effective in inducing root hair curling and nodule primordia. Thus, our results suggest that, to produce the differential expression of these genes, Nod factor perception also is required when wild-type bacteria are hosted inside ITs.

Apparently contradictory results were published for the expression of the *ENOD2* gene during nodulation. Mitra and Long (2004) reported that bacterial mutants affected in their ability to invade the host root (*exoA* and *exoH*) did not induce expression of this nodulin in *M. truncatula*. On the other hand, expression of *ENOD2* in empty nodules was induced by mutants affected in the cyclic glucan synthesis (*ndvB*) but also by an *exoA* mutant in alfalfa (Dickstein et al. 1988). These conflicting findings were explained by the different developmental stages of nodule-like structures induced by these mutants and led to the suggestion that development of symbiosis is more tightly controlled in *M. truncatula* than in alfalfa (Mitra and Long 2004). Here, we described that, during the wild-type interaction with *L. japonicus*, *ENOD2* expression was increased between 7 and 21 dpi, an effect that was not observed during interaction with the *cgs* mutant. This could be an indication that the pseudonodules induced by *cgs* on *L. japonicus* were arrested in an early developmental stage.

Concluding remarks.

Analysis of the transcriptional profile of *L. japonicus* genes upon inoculation with *M. loti* wild-type and mutant strains affected in different steps of the symbiotic process led to the identification of several genes whose expression is related to the nodule bacterial invasion and nodule maturation. Several new genes that previously were associated with the plant-pathogen interactions were found to be differentially expressed, giving new insights into roles of plant defense systems during symbiotic interactions. Components of early recognition and signaling pathways also were found to be altered when using a bacterial mutant strain that is unable to invade the host root;

therefore, these results support links between recognition of symbiotic bacteria and control of infection by classical plant defense systems.

MATERIALS AND METHODS

Plant and rhizobial cultivation.

M. loti strains were grown at 28°C in AB minimal medium (Douglas et al. 1985) supplemented with sucrose (0.5% wt/vol). For growth of mutant bacteria, gentamicin was added to a final concentration of 30 µg/ml.

L. japonicus GIFU B-129 seed were surface sterilized and pregerminated. Three-day-old seedlings were planted onto square petri dishes that contained quarter-strength B&D medium (Broughton and Dilworth 1971) for sterile cultivation in a controlled environment. Plants were grown for 1 week before being inoculated with different *M. loti* strains at an optical density of 0.6 at 600 nm (10 µl/plant). Plants were harvested directly into liquid nitrogen and stored at -80°C.

cDNA array.

Construction, design, and hybridization procedures of these arrays containing 9,600 redundant PCR-amplified EST clones from a nodule library was described previously (Colebatch et al. 2002a; Ott et al. 2005). In all, four replicates representing two independent experiments were performed for each condition analyzed.

Quantitative real-time RT-PCR.

Quantitative determination of relative transcript levels by Real Time RT-PCR was carried out according to Czechowski and associates (2004), except that total RNA was isolated from roots using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Transcript levels were normalized to ubiquitin. Gene-specific primers were designed using the Primer Express Software (Applied Biosystems, Foster City, CA, U.S.A.). Sequences of oligonucleotide primers used in RT-PCR experiments are shown in Supplementary Table C. In all, four replicates, representing two biological replicates, were performed for each condition analyzed. The tentative consensus (TC) repository can be found at the Gene Index Project website.

Competition assay and fluorescence microscopy experiments.

Plasmids pDG71 (containing *gfp*) and pDG77 (containing DsRed) (Gage 2002) were transferred into *M. loti lpsβ2* and *M. loti* wild-type strain by triparental mating. For competition assay, *M. loti lpsβ2*/pDG71 and wild-type strains/pDG77 were mixed together in 1:1 ratio and inoculated on *L. japonicus* plants as described previously (Gage 2002). At different times postinoculation, the occurrence of IT formation was evaluated by using fluorescence microscopy. Fluorescence microscopy experiments were made by using a Nikon ECLIPSE E600 EPI-FL microscope. The filter set for fluorescence microscopy consisted of a 450- to 490-nm band-pass excitation filter and a 515-nm barrier filter for *gfp*-expressing *M. loti lpsβ2* and DsRed-expressing *M. loti* strain was imaged by using a 540- to 480-nm band-pass excitation filter and a 600- to 660-nm barrier filter. At 3 weeks postinoculation, five nodules were harvested, sterilized, and crushed in AB medium as described previously. To determine the relative proportions of the mutant strain with respect to the wild-type strain, serial dilutions were plated on AB sucrose 0.5% minimal medium with or without gentamicin. The experiment was performed in duplicates. The competitiveness index (CI) was calculated as the following ratio: CFU of mutant recovered from the nodule per CFU of total bacteria recovered from the nodule.

The *cgs* mutant strain harboring the pTB93F plasmid (Gage et al. 1996) was used to analyze the formation of ITs on *L. japonicus* as described previously (D'Antuono et al. 2005).

Histochemical assay.

For in situ localization of polyphenols accumulation, plants were inoculated with *M. loti* wild-type and *M. loti cgs* strains. Plants were harvested at different times postinoculation and stained as described previously (Vasse et al. 1993). Briefly, whole plants were fixed with a solution of 10% glutaraldehyde and then postfixed with a solution 0.04% potassium permanganate. After rising, plants were stained for 10 min with a 0.01% aqueous solution of methylene blue and finally cleared for 3 min with an aqueous solution of sodium hypochlorite. The plants were examined by bright-field or phase-contrast microscopy with a Nikon ECLIPSE E600 EPI-FL microscope.

ACKNOWLEDGMENTS

The project was supported by the grant from the Deutscher Akademischer Auslandsdienst Deutscher Akademischer Austauschdienst and from Fundación Antorchas (project number 14116-201) and by a grant from the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2004 N0 20408).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Dana Farber Cancer Institute and Harvard School of Public Health Gene Index Project website:
compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=l_japonicus